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The effects of herbicides on microbial and mycorrhizal populations in native prairie, corn, and non-native prairie vegetation, using the Biolog technique

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THE EFFECTS OF HERBICIDES ON MICROBIAL AND MYCORRHIZAL POPULATIONS IN NATIVE PRAIRIE, CORN, AND NON-NATIVE PRAIRIE VEGETATION, USING THE BIOLOG TECHNIQUE

An Abstract of a Thesis

Submitted

In Partial Fulfillment

of the Requirements for the Degree of

Master of Science

Susann E. Ahrabi-Fard

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ABSTRACT

Soil bioremediation has primarily focused on the use of free living, actively growing microorganisms to degrade toxic organic substances. Recently, however, plants and rhizosphere activity, including mycorrhizal fungi have been increasingly studied to determine the role that consortial assemblages may play in bioremediation. While the enhanced absorbtion of nutrients, water, and minerals by mycorrhizal fungi has been well documented, less is known about their role in the degradation of soil contaminants. Using the Biolog plate technique, microbial communities were characterized in Native Prairie, Corn, and Non-Native Prairie vegetative strips prior to, and after two applications of Acetachlor, Alachlor, and Atrazine. Mycorrhizal hyphae growth was also examined under increasing concentrations of the same three chemicals. Microbial biomass was affected by herbicide application among the vegetative types in the test strips. Mycorrhizae grown on various concentrations of herbicides also showed decreased growth as herbicide concentrations increased.

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A sole-carbon-utilization technique for bacterial characterization is now being applied to environmental sampling situations. This method, called the Biolog assay (BIOLOG Inc., Hayward, CA), uses the reduction of a tetrazolium dye indicator in 95-well microplates to characterize the potential activity of a community.

The purpose of this research was to evaluate the effect of herbicides on microbial populations in field samples, using the BIOLOG assay. Microbial communities were characterized, using BIOLOG microplates, in Native Prairie,

Corn, and Non-native Prairie vegetative strips prior to, and following two applications of an Acetachlor, Alachlor, and Atrazine mixture.

The growth of mycorrhizal hyphae under increasing concentrations of the same herbicide mixture was also examined. The enhanced absorption of nutrients, water, and minerals by mycorrhizal fungi has been well documented, but less is known about the affects they have in chemical transport to the plants they infect. The role mycorrhizal fungi play in the degradation of soil contaminants is also a growing area of interest.

Although varying patterns were seen between corn and prairie grasses, the BIOLOG method did not seem sensitive enough to detect small changes in population shifts after herbicide application. Large variations were also not observable in microbial populations among the different vegetative types. Time and biomass were discovered to be important factors to consider when using this technique. Bacterial numbers were lower in areas of herbicide application when evaluated by a bacterial enumeration technique.

Mycorrhizae grown on various concentrations of herbicides showed decreased hyphal growth and spore germination as herbicide concentrations increased.

LITERATURE REVIEW

The use of plants and microorganisms to remediate the environment and as a tool in pollution prevention is an expanding area of interest. This method known as "Bioremediation," covers a wide array of processes varying in complexity from the simple act of stimulating pre-existing organisms to using complicated technology to achieve desired results.

Various organisms have been found to be effective degraders of organic pollutants, including plants, fungi, and most commonly, bacteria. Use of these organisms *in situ* has often proven to be a cheaper, more effective, and a less intrusive method of clean-up than alternatives such as physical removal, and/or incineration of contaminated materials. *In situ* bioremediation generally involves enhancing bacterial activity by improving the environmental conditions, i.e., by adding nutrients, electron acceptors, or moisture (Bollag et al., 1994). The effectiveness and cost efficiency of bioremediation has led to its increased use and application in varied environmental situations.

Bioremediation may be limited in an area if indigenous microorganisms are unable to metabolize the pollutants. Microorganisms only metabolize specific organic compounds, and minor compositional changes may not be subject to initial attack by the same microbial enzyme. Additionally, pollutants often are mixtures of various chemicals, such as oil, polychlorinated biphenyls and agricultural products. These mixtures have different effects on microbial degradation capabilities and may be toxic (Atlas, 1995). Rates of degradation are also greatly affected by environmental conditions such as temperature, moisture, pH, oxygen and nutrient availability. Thus, each contaminated site must be characterized and a bioremediation plan must be designed to fit that specific site (Atlas, 1995).

Most bioremediation techniques work by enhancing natural detoxification of polluted environments through stimulating the activity of indigenous microorganisms using techniques such as the addition of nutrients, optimization of pH conditions, and regulation of redox conditions. Future techniques may include inoculating contaminated areas with microorganisms or enzymes capable of specific

biotransforming abilities, and the use of plants to contain or transform both organic and inorganic pollutants (Bollag, et al., 1994). Heavily contaminated sites may create environments which kill indigenous organisms, or are polluted with recalcitrant xenobiotics. These areas could benefit from inoculation methods. By using gene transfer techniques, or culture enrichment, large populations of xenobiotic-degrading bacteria could be produced, and then inoculated into a contaminated area. This method would best be suited to areas that are highly contaminated, possibly areas bereft of competitors and predators. In a less polluted soil, the introduced bacteria would have to compete for resources with the indigenous organisms, and avoid predation (Bollag et al., 1994). Modifying microorganisms at the molecular level to improve degradative abilities, and genetically engineering organisms with unique degradative characteristics will be large areas of interest in the future. For example, in Japan a research group has isolated a *Pseudomonas* species that can grow in solvents containing over 50% toluene. This group is attempting to add appropriate genes for catabolic enzymes which will

potentially expand the range of bioconversion into nonaqueous solvents, by this organism (Atlas, 1995). Another modification, described by Atlas (1995), is being used by Sayler at the University of Knoxville, Tennessee. By using the expression of the lux gene which codes for bioluminescence, Sayler has attached it to the genes coding for naphthalene degradation. The lux gene allows the degradation of naphthalene to be monitored through the observation of the bioluminescence (Atlas, 1995). Research to better understand bioremediation processes, and decrease drawbacks will be areas of large scale interest in the future.

With the potential for bioremediation to be a widely used clean-up method, it is important to have many alternative methods of treatment to compensate for the high specificity of microorganisms. A wide array of organisms, and various ways to use them will allow researchers to create clean-up strategies by picking organisms which are best suited for specific pollutants, under certain environmental conditions, in differing types of soil or substrate. Expanding bioremediation research to other

organisms, such as plants and fungi, greatly increases the range of clean-up methods available.

Bacterial Bioremediation

In the past, bioremediation primarily focused on degradative abilities of microbial populations. Soil bioremediation has been occurring naturally since the beginning of life. There are a vast array of naturally occurring toxic and recalcitrant organic compounds on the planet which are naturally converted to carbon dioxide. Microorganisms are responsible for the mineralization of most organic matter, with matter that is not readily mineralized being incorporated into humus. Using a naturally occurring mineralization process to clean up man-made pollutants is the key to bioremediation, and involves the understanding of microbial ecology, soil chemistry, soil biology, and engineering (Bollag et al., 1994).

The presence of microbial populations is important in the ecology of the soil, nitrogen-fixation, and CO_2 production. Bacteria have been known to be effective at degrading environmental pollutants aerobically, and anaerobically in soil and aquatic environments for some

time. They have been successfully used in the treatment of municipal and industrial wastewater, and along with agricultural waste can be treated in various ways such as waste lagoons, bioreactors as activated sludges, fixed media reactors using trickling filters, and fluidized bed reactors. With over 1200 Superfund sites awaiting clean-up, and the United States generating about 300 million metric tons of hazardous waste a year, the use of microorganisms is being expanded to include large scale treatments (Bollag et al., 1994).

Rhizosphere Remediation

Interactions between plants and microorganisms in the rhizosphere are complex and have developed to be of mutual benefit to plants and their associated microflora.

Soil communities as a whole, are now being studied to better understand bioremediation. These communities include bacteria, protozoa, plants, fungi, nematodes, actinomycetes, as a working unit in the rhizosphere.

The rhizosphere is an area surrounding active roots, where there is elevated microbial activity and population. It is this increased activity and biomass that distinguishes

the rhizosphere from bulk soil (Curl, 1986). This area is at the root-soil interface and is under the influence of the plant root itself. The plant roots serve as a colonization surface for various microorganisms (Curl, 1986; Atlas, 1992; Bolton, 1993). Release of organic substrates from the roots, allow higher numbers of microorganisms and fungi to survive in this area (Garrett, 1981). Root systems are continuously releasing metabolic substrates including sugars and amino acids. This occurs through the secretion of exudates and root turnover. Root cap cells which protect the root from abrasion can be lost at a rate of 10,000 cells per plant a day (Campbell, 1985). Root cells also excrete a substance known as mucigel, which is a gelatinous lubricant for root penetration through the soil during growth. Root cap cells and exudates provide important nutrients that microorganisms can use for carbon and energy sources, and in turn, the products of microbial metabolism stimulate plant growth. Once microbial populations have been established in the rhizosphere of plants, they can be passively nourished by root exudates and decaying plant matter (Rovira et al., 1979). Some microorganisms have developed the ability to

produce biochemical signals that induce the release of organic nutrients from the plants, perhaps through co-evolved biochemical signals. Two bacteria, *Pseudomonas putida* and *Azospirillum* species, are capable of inducing the release of nutrients from plant roots (Prikryl, 1980).

Rhizosphere microbial communities tend to be predominantly gram-negative, with many parameters affecting the community make-up. Influencing factors include oxygen, CO_2 concentrations, osmotic and redox potentials, soil pH and moisture content (Curl and Truelove, 1986). The amount and type of exposures to chemical pollutants also has an influence on the rhizosphere community (Sandmann, 1984). The effect of the rhizosphere can be quantitatively expressed as a ratio of microorganism numbers in rhizosphere soil to the number of microorganisms in non-rhizosphere soil (R/S ratio; Katznelson, 1946). An R/S ratio will normally run from 5 to 20, but can be as high as 100 or more (Atlas, 1992). It has been observed that plants grown in the absence of bacteria and fungi have a decreased exudate secretion compared to those grown in the presence of bacteria and fungi (Curl, 1986). Interactions present in

the rhizosphere result in increases of microbial biomass by an order of magnitude or more, compared with bulk soil microbial population. The actual composition of a rhizosphere community depends on many factors including plant species and age, root types, and soil type (Campbell, 1985; Atlas, 1992; Bolton, 1993). For example, the root systems of grasses tend to be large and fibrous, serving as a better colonization area for microorganisms than taproot systems (Atlas, 1992). The elevated activity and biomass in the rhizosphere area could be responsible for increased metabolic degradation of chemical pollutants. Selection for plants possessing proliferation of root hairs, supernodulation roots or other genetic properties affecting the size of the rhizosphere zone could then also influence the rates of microbial degradation in that area (Anderson, et al., 1993). Degradation of organic contaminants, especially insecticides and herbicides, by microbial consortia has been studied and has repeatedly shown that microbial numbers are elevated in areas exposed or treated with organic chemicals, versus untreated areas. It has been proposed that the high concentrations of microbes within the

rhizosphere and their unusual catabolic capabilities could be manipulated to enhance degradation or toxic organic compounds. Two basic methods for enhanced metabolism are under investigation (U.S. Dept. of Energy, 1994.) The most common exploitation of rhizosphere-enhanced degradation is simply to increase the root biomass of soil at contaminated sites. Many soils have been abused through years of contaminant application and are unable to support a complex and diverse community of plant species. Without the presence of a large concentration of roots, degradation within the soil is limited. An important key to remediation in this type of area is to establish a healthy and viable crop. This is accomplished by improving the soil's physical and chemical environment, often with the application of bestialities or animal manures being sufficient. If multiple contaminants are present, the crop planted on the site must be resistant to all of the contaminants found at the location. The crop is then managed for maximum production of biomass. Land farming, as this is called, is currently the most effective method for remediating a

variety of organic contaminants (U.S. Dept. of Energy, 1994).

The second enhancement technique being evaluated is that of using plants to preferentially select for microbial species that can survive within the rhizosphere. Although little work has been conducted in this area, it is well known that certain species of bacteria can preferentially survive within the rhizosphere. By transferring catabolic genes into the rhizosphere bacteria, it is possible to overcome the most important limitation affecting bioremediation--the inability of the introduced catabolic organism to survive within the soil. Angle et al. (1995) have shown that survival in the rhizosphere is enhanced, with introduced organisms surviving for many months longer than organisms in bulk soil. The potential exists to create "biased rhizospheres" where a plant root exudate favors the growth of one type of microorganism that can subsequently be selected or engineered to degrade specific organic pollutants (U.S. Dept. of Energy, 1994). In the future, manipulation of rhizosphere communities may serve to enhance the degradation of contaminants.

Phytoremediation

Green plants are under continual assault by a wide variety of naturally occurring toxins of fungal, plant, and bacterial origin. To survive, plants have evolved sophisticated metabolic and sequestration mechanisms to detoxify various chemical substrates. Not all plants, however, are equally capable of surviving and detoxifying anthropogenic chemicals, thus not all sites will prove amenable to using plants for detoxification. Plants have significant differences in metabolic capability among genera, species and even cultivars. These differences are perhaps best realized as the basis of the development of most modern herbicides. Tolerant plants are able to metabolize applied toxins, while intolerant plants lack the metabolic ability and die. The potential of a plant to metabolize toxins is not, however, inflexible. It evolves naturally, and can also be altered using lab techniques (Cunningham, 1995 conference).

It is now commonly shown that plants have a large potential in the containment and removal of organic and inorganic pollutants in the environment. Plants have

successfully been used for wastewater treatment for many years, and are now being studied for use as agricultural buffer strips (Bollag et al., 1994). Shnoor, at the University of Iowa, has been testing poplar trees, which are fast growing and have a deep root system, as a buffer strip between landfills or agricultural fields and waterways (Personal Communication, 1995). Grass and fescue filter strips have been tested for their ability to trap or decrease the concentrations of soil and fecal coliforms (Coyne et al., 1995) and swine manure (Edwards and Daniels, 1993) with some success.

Plants can also concentrate organic and inorganic pollutants from soils to harvestable portions of the plant. Pollutants can then be removed by removing the plant. Metabolic processes in some plants can then mineralize or transform the pollutant. The metabolic diversity of plants suggests that xenobiotic organics can be degraded by at least some species and evidence is accumulating that plants have a role in the degradation of persistent organic contaminants. Phytoremediation of organics depends not only on the ability of the compound to be metabolized or

irreversibly isolated, but its ability to be transported to the plant root and the root-associated micro flora (Cunningham, 1995 conference).

Plants have natural attributes that make them ideal candidates for remediating contaminated soil environments. The root system represents an enormous surface area that enables plants to absorb and accumulate the water and nutrients essential for growth. Plants have remarkable metabolic and absorption capabilities and possess transport systems that can selectively take up many ions from soils. Plants have evolved a great diversity through genetic adaptations to help them deal with potentially toxic levels of metals and other pollutants that occur in the environment. Most metal tolerant plants exclude toxic metal ions from uptake, while "hyperaccumulators," actually tolerate and take up high amounts of toxic metals and other metal ions, up to several percent of their dry matter weight (U.S. Dept. of Energy Workshop, 1994).

Applications of phytoremediation include buffer zones for the control and treatment of leachates, bioremediation of organics, and the stabilization of soils, dusts and

metals. Plants root systems can do the actual "pumping and treating" of pollutants, with energy being provided by the sun. This alleviates the need for machinery to pump or shovel contaminated soils or water up to the surface. Root systems can transport oxygen down through the soil to the pollutant, where the pollutants can be completely degraded, possibly in the rhizosphere, to carbon dioxide (Larry Erickson, personal communication, 1995).

Future areas of research in the field of phytoremediation will need to focus on areas such as mechanisms of uptake, transport, and accumulation, the genetic analysis of rhizosphere interactions, hyperaccumulating plants, and field evaluation and validation (U.S. Dept. of Energy, 1994).

Fungi in Bioremediation

Fungi are the second most prevalent group of microorganisms in soil, sometimes even surpassing bacterial biomass in a given area. There are three main types of fungi present in soil: pathogenic root-infecting fungi, saprophytic fungi, and mycorrhizal fungi (Walton et al., 1994).

Interest has developed in the use of various fungi, such as mycorrhizal and white rot fungi, as a soil inoculum for the degradation of pollutants. Soil fungi, such as *Aspergillus fumigatus,* are known to be useful in the degradation of s-triazine herbicides (Kaufman and Blake, 1970). White rot fungi, such as the basidiomycete *Phanerochaete chrysosporium,* can degrade a large range of aromatic organopollutants. This species and other white rot fungi, have the ability to degrade lignin, due to the presence of two extracellular enzymes: lignin peroxidases and Mn-dependent peroxidases. The degradation of lignin is necessary for the decomposition of wood in nature, and is one of natures most persistent organic polymers. The ability to degrade lignin by fungi, led researchers to look for the potential to degrade other environmental contaminants. Research has shown that the lignin-degrading system of these fungi are very effective degraders of a wide range of pollutants, including polychlorinated biphenyls, TCDD, DTT, azo dyes, phenanthrene, and other polycyclic aromatic hydrocarbons in laboratory situations (Tien and Myer, 1993). Since this discovery, there has been wide-

spread interest in the use of *Phanerochaete chrysosporium* for clean up of contaminated soils.

Mycorrhizal fungi have gained a lot of attention for their ability to degrade pollutants in the rhizosphere. Mycorrhizal fungi form a mutualistic, symbiotic relationship between plant roots and fungal mycelia, which create unified physiological entities (Walton et al., 1994). Mycorrhizal fungi depend on root interaction to grow and reproduce, otherwise remaining in a dormant state as spores or resistant hyphae. Mycorrhizal fungi differ in their specificity, with certain species having a broad host plant range, while others are only able to infect a few species of host plant. Plants also differ in their need for the mycorrhizal-root interaction. Some plants can have numerous mycorrhizal species infecting their root systems at any given time (Nester, 1978).

There are two general categories of mycorrhizal fungi which is based on the position of the hyphae in relation to the root epidermis of the host plant. **Ectomycorrhizae** roots are usually swollen looking, appearing considerably more forked than non-mycorrhizal roots. Ectomycorrhizae do not

penetrate the host plant cortical cells, but form a hyphal network, or tightly interwoven fungus mantle, surrounding feeder roots. This dense sheath acts to increase the surface area for absorption (Rendig and Taylor, 1989). The hyphae also enter the roots, but only grow around the cortical cell, replacing part of the middle lamella between the cells, forming a "Hartig net." Spores of ectomycorrhizae are produced above ground and are wind disseminated. This association is very significant in mineral-deficient soils, and is most common in forest trees (conifers) such as pine and spruce (Nester, 1978).

Mycorrhizal fungi that penetrate into the cortical cells in the feeder root of the host plant are known as **endomycorrhizae**. This type of root tends to look like non-mycorrhizal roots in shape and color. Endomycorrhizae are not surrounded by a dense fungle mantle, but by a loose mycelial growth on the root surface. Hyphae and zygospores or chlamydospores are produced underground from this root surface. Inside the cortical cells of the feeder root, highly branched structures, called arbuscules, act as specialized feeding hyphae. The arbuscules are digested by

the plant, releasing nutrients inside. Large, swollen food-storing hyphal swellings, called vessicles, may also form. Most endomycorrhizae contain both of these structures, and are thus called "vesicular-arbuscular" (VA) mycorrhizae (Nester, 1978). This type of mycorrhizae is more common in cultivated plants such as corn, wheat, and deciduous trees (Rendig and Taylor, 1989).

Plants benefit from the root-mycorrhizae interaction in numerous ways. Plants involved in the symbiosis display greater drought tolerance, resistance to soil-borne diseases, and reproductive fitness. Plants seem to have better access to nutrients due to the ability of the fungi to acquire inorganic nutrients (minerals) from the soil and translocate them to the plant. The fungi obtain nutrients from the host plant, and the plant receives extra nutrients through the increased surface area provided by the fungi. It is also believed that the host plant provides a soluble carbon source to the fungi (Donnelly and Fletcher, 1995). Soil health is improved by mycorrhizae through increased soil tilth and soil aggregation, and improved anchoring and growth for plant roots, which decreases erosion. Resistance

to potentially toxic substances and pathogens may be due in part to the physical barrier of the root by the fungal hyphae. Fungal secretions, such as organic acids, antibiotics and detoxification enzymes may play a role in this. It is this last benefit that environmental scientists have been investigating, to understand the role mycorrhizal fungi play in detoxification and degradation of environmental contaminants.

The prospect of mycorrhizal fungi existing in nature has important implications for bioremediation because mycorrhizal fungi introduced in combination with their host plant at a contaminated site may survive and have sustained degradative properties, features not often characteristic of bacteria (Donnelly and Fletcher, 1995). Several species of ectomycorrhizal fungi have been shown to produce the enzymes necessary to degrade complex aromatic compounds in the soil. The presence of these enzymes indicate that mycorrhizal fungi may not be completely dependent on the host plant for its carbon supply. By producing these enzymes, they also have the potential ability to utilize other carbon sources found in the soil ecosystem and may play an important role

in nutrient cycling. Whether this actually occurs needs to be studied, but studies show that this group of fungi are capable of degrading a wide range of compounds (Donnelly and Fletcher, 1995).

Donnelly et al. (1993) found that several mycorrhizal fungi were capable of degrading chlorinated aromatic herbicides like 2,4-D and atrazine, by incorporation of herbicide carbon into tissue, but not mineralization. In the same study, several mycorrhizal species were also shown to metabolize polychlorinated biphenyls to varying degrees (Donnelly et al., 1993). Some herbicides can greatly influence arbuscular fungi and mycorrhizal formation (Trappe et al., 1984). The influence can be direct, by affecting the host plant and actually inducing mycorrhizae formation as in the case the herbicide simazine, on *Chenopodium quinona*, an otherwise non-mycorrhizal species (Schwab et al., 1982). The influence of herbicides can also be negative, decreasing or preventing the colonization of mycorrhizae on roots. Hamel et al., (1994) conducted an experiment that looked at herbicide application after mycorrhizal establishment in apples. The study showed that

there was an increase in the toxic response of apple rootstocks treated with simazine, paraquat, and dichlobenil when plants were mycorrhizal. It was also seen that the percent root colonization was not significantly affected, but the size of the mycorrhizal root systems was reduced. At the highest rate of simazine treatment, mycorrhizal plants died, while none of the non-mycorrhizal control plants died. Death of the apple plants, accompanying a lack of effect on root colonization and hyphal elongation, suggest that the herbicides tested were more toxic to the apple plants than to the mycorrhizal fungi, and that the mycorrizae increased the phytotoxicity of the chemicals by improving the uptake by apple rootstocks (Hamel, 1994). This research supported findings of Nelson and Khan (1992), who demonstrated that mycorrhizal hyphae were able to remove atrazine from soil and transfer it to plants (Nelson and Khan, 1992).

It is believed that if a mycorrhizal-root relationship is good, less chemical fertilizer will be needed for optimum growth of host plants, however reduced populations of mycorrhizal fungi, and low levels of root colonization are

found in conventional agricultural fields today. This suggests that either this interaction is not functional under standard agricultural procedures (tilling), or is decreased as a result of continuous and heavy chemical application (Interdisc. Plant Group--14th Ann. Symp., 1995).

The biggest difficulty in measuring mycorrhizal activity is growing the fungi in a lab setting. Despite increasingly intensive study of VA mycorrhizae, many areas are still poorly understood. Areas of interest include: mechanisms by which spores remain dormant in soil in the absence of plant roots, triggering of spore formation, how germ tubes locate roots, and means of infection, spread, growth and sporulation (Koske, 1981). It is possible to grow mycorrhizae in pot cultures, and to transfer them to agar, but spore formation does not occur on agar plates, only hyphal growth. The absence of spore formation in lab settings is a limiting factor in the amount of information that can be gained about mycorrhizal fungi.

It should be emphasized that research about chemical degradation to date has been done using *in vitro* studies without host plants. Mycorrhizal fungi may not metabolize

these compounds to the same extent when growing with host plants. While studies with the intact symbiosis are in progress, more research needs to done. Enzymatic and physiological studies completed with mycorrhizal fungi indicate that these fungi have the potential to play a role in the bioremediation of hazardous compounds in the soil. The natural, sustained occurance of this interaction provide an ecologically sound approach to long-term clean-up and ecological restoration of contaminated sites (Donnelly and Fletcher, 1995).

Measuring Soil and Rhizosphere Activity Using the BIOLOG Technique

Measuring microbial consortia and rhizosphere activity in soil is very difficult due to the complexity of the communities involved. Microbial ecologists have not been very successful at identifying and classifying microbial communities due to the small size, and morphological similarity of constituent members. Most studies of heterotrophic microbial community structure have involved enrichment and selection-based methods. Numerical taxonomic studies generally use profiles of cellular constituents or phenetic characteristics of isolates to define taxonomic

units. Analysis of phenotypic characteristics relating to processes in the environment, and correlation of such characteristics to environmental parameters give greater insight into factors controlling the structure of a community. Isolate-based methods are dependent on culturing methods, and may exclude many indigenous microbes due to the exclusive nature of the media. In addition, the time consuming nature of isolate-based methods limits the spatial and temporal intensity of sampling (Garland and Mills, 1991).

Microbial (bacterial and fungal) population shifts, *in situ* is difficult to assess because methods for testing have not been developed that can reliably analyze microbial community structure and function. In analyzing biological communities *in situ*, many factors must be considered, such as species diversity, activity and function. Techniques involving the cultivation of microorganisms on agar plates have the disadvantage of only sampling a small portion of the community, due to the fact that many soil microorganisms are as of yet, unculturable with modern day methods (Bakken, 1985). Studies using plate count methods have been able to

show population shifts in species of bacteria in metal-contaminated soils (Barkay, 1985).

It is possible to use techniques such as radiorespirometry to examine potential activity, and phenotypic and genotypic characterization to identify specific organisms in mixed microbial communities. However, little is known about population dynamics indicating a need for methods that can give insight into community interactions. Use of community-level approaches to observe microbial function and activity would provide a more sensitive and ecologically meaningful measure of heterotrophic microbial populations. Rather than relying on determination of changes in individual abundances, which may not equate to meaningful shifts in community function, the community-level approach would provide measures of the metabolic abilities of the community (Garland and Mills, 1991).

White and Findlay (1988) have developed a community-level method for characterizing microbial community structure by looking at shifts in fatty acid methyl esters from whole environmental samples. The method has proved

successful in detecting changes in biomass of total bacteria, diatoms, and microeukaryotes in various microbial habitats. The method does eliminate the bias associated with cultural methods, and increase the scope of sampling, but structural markers for shifts within the heterotrophic bacterial community still need to be developed. The analysis of taxonomic structure of communities alone, however, limits insight into the ecological relevance of the community structure (Garland and Mills, 1991). Community shifts and impacts caused by pollution, agricultural practices and ecosystem management can have great impacts on the dynamics of a population. Interest in quantifying the impacts of various factors on microorganism communities in soil environments has increased as concern about agricultural and industrial pollution has become more prevalent (Bossio and Scow, 1995).

Biochemical techniques, such as characterizing phospholipid fatty acids (PFLA), can help characterize the structure of microbial communities in the soil. Testing with PFLA has mostly been used to test microbial communities in lake and seawater, and in studies of biofilms and

sediments. Only a few studies have used PFLA to detect changes in the community structure after environmental disturbances in soil (Frostegard et al., 1993). While a method such as PFLA can give more insight into entire community responses and shifts in community composition than more traditional methods such as plate counts, it is generally not useful for detecting individual strains or species of microorganisms (Frostegard et al., 1993).

In an attempt to find a method for examining community composition and response to perturbations, Garland and Mills (1991) proposed sole-carbon-source utilization could be used as a functionally based measure for classifying heterotrophic microbial communities. A community-level assay of community structure based on carbon source characters would require rapid multiple assays of carbon source utilization. An oxygen utilization microplate technique has been developed recently by BIOLOG Inc. (Hayward, CA) for carbon source utilization testing of bacterial isolates for strain identification. These microplates, which are used clinically for identification of isolates, are now being applied to environmental testing

methods. The BIOLOG assay is done by visually or colorimetrically measuring tetrazolium dye reduction, which is coupled to substrate oxidation (oxygen depletion), and the number of the 95 substrates oxidized determined after a fixed incubation period. If the values for all substrate-containing wells are analyzed by multivariate statistics, two types of distinctions between samples can be made. First, the presence or absence of a positive response to each of the 95 substrates or to groups of substrates, such as polymers, can be recorded. Secondly, when the same substrates are utilized by different communities, samples with consistently high values for certain substrates can be segregated by multivariate analysis from those with consistently lower values for the same substrates. Zak et al. used both of these to successfully distinguish between desert soil communities (Zak et al., 1994).

The ability to rapidly visualize community structure as a composite of functional abilities (or potentials) enables both the extensive comparison of microbial communities across a wide range of spatial and temporal variables, and the identification of ecologically relevant functional

differences among communities (Garland and Mills, 1991). Garland and Mills found the BIOLOG method useful in the separation of microbial communities in soil and water samples. BIOLOG plates were inoculated with environmental samples from freshwater, saltwater, estuarine, soils, and hydroponic solutions from the rhizosphere of hydroponically grown wheat. They found the BIOLOG community assay to be rapid and sensitive enough to conduct the intensive sampling required to examine the mechanisms coupling sources of bacterial substrates to dynamics in the bacterioplanktonic community in aquatic systems. Their research also indicated the direct inoculation of environmental samples into BIOLOG plates produced patterns of metabolic response useful for classification and characterization of microbial communities. Similarly, Winding (1994) analyzed whole-community BIOLOG substrate utilization profiles of samples taken from several types of forest soils, along with those from size class fractions within one soil type (Winding, 1993). Zak et al. (1994) also used the BIOLOG technique to assess the functional diversity of microbial communities associated with six plant communities (Zak et al., 1994).

In a recent study by Bossio and Scow (1995), it was shown that the BIOLOG method was effective at showing the impact of carbon on the metabolic diversity of soil microbial communities, and that differences among microbial communities in their BIOLOG sole-carbon-source utilization patterns could be explained by the environmental variables imposed on the communities. The authors felt the method also exhibited seasonal fluctuations in direct correspondence to the environmental fluctuations, showed similar patterns at two independent locations, and the ease of using the BIOLOG technique made it feasible for large scale field studies (Bossio and Scow, 1995). Conversely, the method requires organisms to be metabolically active in solution cultures under conditions that differ greatly from their natural environment, thus the methods cannot be expected to reflect the metabolic capabilities of the entire community (Bossio and Scow, 1995).

As pointed out by Haack et al. (1995), Winding (1994), Garland and Mills (1991), and Zak et al. (1994) in addition to establishing ecologically relevant classifications of microbial communities, substrate utilization profiles may

offer information with regard to community function (Garland and Mills, 1991), metabolic potential (Winding, 1994), or functional diversity (Zak et al., 1994). Investigation of various factors affecting substrate utilization patterns have found the patterns highly reproducible for model bacterial communities (Haack et al., 1995). To interpret the metabolic potential or functional diversity of microbial communities based on BIOLOG patterns of substrate utilization, it is necessary to understand factors which affect substrate utilization when the microplates are inoculated with microbial mixtures. It is easy to assume that pattern differences of BIOLOG substrate oxidation by different communities represent real variances in the numbers or types of organisms in these communities, and that these variances reflect the activities expressed in the environments from which the samples were obtained. However, both Garland and Mills (1991), and Winding (1993) found that variation in response from one sample to another was not necessarily a function of structural differences in the communities. Instead, they both noted strong correlations between the inoculum cell density and the rate of color

development. This suggested that community differences in substrate oxidation recorded at a fixed time may only reflect differences in the total number of organisms in the communities. Both groups also found that color development appeared to depend on cell growth in the substrate-containing wells. This made it impossible to determine if all members of the community capable of utilizing the compound had contributed to the profile, or if the response in a given well resulted from growth and activity of a few or only a single member of the consortia (Haack et al., 1995). These limitations were acknowledged by Zak et al. (1994), but is was argued that substrate utilization profiles were still capable of providing a "rich data set" with which to study the function and diversity of microbial communities. It was further proposed by Garland and Mills (1991) that more research was required to determine whether community-level carbon substrate utilization patterns were repeatable, and to determine the causes of differential substrate oxidation among samples. The BIOLOG technique has the potential to be a useful tool in the characterization of microbial communities, especially as a monitoring tool to

determine differences following environmental perturbations, but reservations exist as to the extent and accuracy with which they characterize microbial communities.

The presence of microbial and mycorrhizal populations is a factor in the degradation of agricultural chemicals that are applied annually. Knowing how microbial community structure in a field may be affected by the addition of chemicals can give important insight into the overall environmental effects of this practice. If significant changes or decreases in microbial populations are seen after herbicide applications, it would suggest that the natural balance and activity of indigenous microorganisms in the soil is harmed by the use of farm chemicals, and may not be in the best interest of the land for sustainable farming.

Purpose of the Project

The overall objective of this study was to characterize microbial consortia in soil, among different plant species, prior to and after the application of herbicides. The BIOLOG technique was used as a rapid community analysis method for measuring population shifts of soil microbial consortia. The effect of exposure to varying concentrations

of three herbicides on hyphal growth of mycorrhizal fungi was also examined using plate-culture assays.

Goals

1) Use the BIOLOG technique to determine whether microbial and\or mycorrhizal populations vary in species composition among vegetative filter strips containing different plant species.

2) Use the BIOLOG technique to determine whether microbial composition changes in soil samples before and after the application of Atrazine, Alachlor, and Acetachlor in soils containing different plant species.

3) Determine the extent to which mycorrhizae hyphal growth is affected when grown in pure cultures containing various concentrations of Atrazine, Acetachlor, and Alachlor.

MATERIALS AND METHODS

Field Site

A test strip of approximately 750 m^2 was located in an agricultural research field, north of the UNI Dome, on the University of Northern Iowa campus. Within the strip were six vegetative filter strips (VFS), two strips each of corn, native prairie grass, and non-native prairie grass species.

Filter strips were 15.2 x 1.8 m. And arranged from left to right as follows: corn, two non-native grass strips, corn, and two native grass strips. Prairie grass strips were established in the spring of 1994. The Corn 1 strip was designated at the same time, but not seeded until the summer of 1995. The Corn 2 strip was added June 8, 1995. Vegetative strips were divided into four quadrants, with the middle two quadrants serving as a buffer between the control quadrant and the quadrant receiving herbicide application runoff. Located between filter strips were 0.9 m buffer zones, lined with polyurethane barriers to prevent cross gradient water flow. A 9.0 m buffer zone of corn lined the sides of the outermost vegetative strips, and a 12 foot buffer zone of corn crossed the ends of the vegetative

strips on one end. The end opposite to that was the drainage end (Fig. 1).

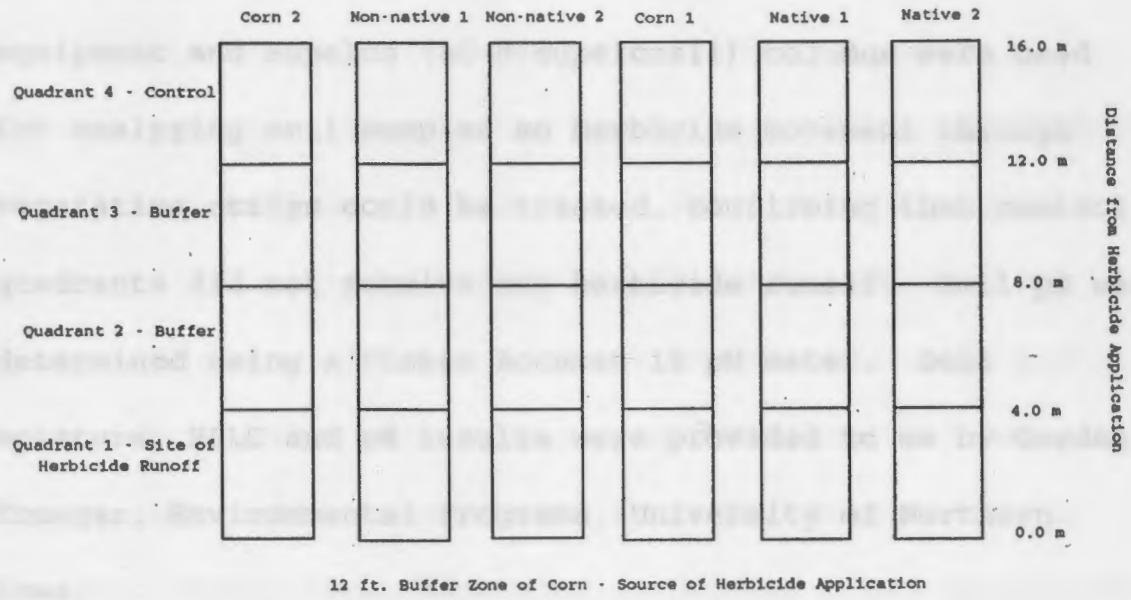


FIG. 1. Schematic of test strip. A herbicide mixture of Atrazine, Alachlor, and Acetachlor was added to the buffer zone. The buffer zone was then flooded with 2,000 gallons of water which was allowed to run into the vegetative strips. The middle two quadrants served as a buffer between the quadrant receiving herbicide runoff and the control quadrant.

Prior to, and during runoff testing, soil moisture content for all the strips was determined. This was done with gypsum block sensors buried at shallow depths within the active root zone of all strips (Native, approximately 1.0 m

and 2.8 m, Non-native, approximately 0.5 m and 1.0 m) and with a soil moisture analyzer (Delmhorst, Model KS-D1). Waters High Performance Liquid Chromatograph (HPLC) equipment and Supelco (LC-8-supelcosil) columns were used for analyzing soil samples so herbicide movement through vegetative strips could be tracked, confirming that control quadrants did not receive any herbicide runoff. Soil pH was determined using a Fisher Accumet 15 pH meter. Soil moisture, HPLC and pH results were provided to me by Gordan Krueger, Environmental Programs, University of Northern Iowa.

Plant Species

Corn and several prairie grass species were planted in the vegetative filter strips. The non-native strips were seeded with fast growing *Bromis inermus* and *Festuca arundinacea* (var. Kentucky 31) grasses. The native grass strips were seeded with a mix of *Andropogon gerardii, Sorghastrum nutans, Elymus canadensis, Panicum virgatum*, and *Schizacyrium scoparium.* The two remaining vegetative strips and surrounding buffer zones were seeded with corn (*Zea maize*).

Chemical Application

Herbicides used in this research were Atrazine (0.84 kg/L active ingredient in solvent), Acetachlor (0.48 kg/L active ingredient in solvent), and Alachlor (0.48 kg/L active ingredient in solvent; Monsanto Inc., St. Louis, MO). The first herbicide application on July 16 consisted of 29.57 mL (1 oz.) of each herbicide solution added to 15 gallons of water (250 ppm Atrazine and Alachlor, 438 ppm Acetachlor). This mixture was sprayed with garden hoses on the 12 foot buffer zone of corn preceding the vegetative strips. Garden hoses were used to deliver 2,000 gallons of water onto the buffer zone creating water runoff into the first quadrant of the vegetative strips, simulating a rainfall event. The second application rate was also a mixture of the three herbicide solutions, composed of twice the typical application rates for active ingredients and crops involved (402 ppm Atrazine, 528 ppm Acetachlor, and 670 ppm Alachlor) in 15 gallons of water. The second herbicide mixture was applied to the corn buffer zone on August 22nd, and the buffer zone was again flooded with

2,000 gallons of water. For details of herbicide application, see Krueger (In preparation).

Soil Sampling

Soil samples were obtained from the field site three times during the summer of 1995 (May 31, July 23, and August 29). Triplicate soil samples were taken at seven distances (0.0, 1.0, 2.0, 4.0, 8.0, 12.0, and 16.0 m) in each vegetative strip, using a 3/4 inch diameter soil corer which was rinsed with 10% hydrogen peroxide solution between samplings. The three samples at each distance were combined in sterile plastic sampling bags, plant debris and visible roots were removed, and soil was then manually homogenized, passed through a 3 mm screen, and stored at 4 °C until use.

BIOLOG Sample Preparation

An oxygen utilization microplate technique that was developed by BIOLOG Inc. (Hayward, CA) was used for carbon source utilization testing of bacterial consortia in soil samples. BIOLOG plates were made up of 95-150 μl cell wells, each containing a different carbon substrate and a tetrazolium dye indicator (Fig. 2). Three "Gram positive" BIOLOG plates were inoculated with each soil sample

collected. A 1:100 slurry of soil in Ringer solution was prepared for each composite sample on collection day. Ringer solution was used for soil slurries because it has the same osmotic pressure as bacteria, and does not supply them with a carbon source.

A1 water	A2 α-cyclodextrin	A3 β-cyclodextrin	A4 dextrin	A5 glycogen	A6 inulin	A7 mannan	A8 tween 40	A9 tween 80	A10 N-acetyl-D-glucosamine	A11 N-acetyl-D-mannosamine	A12 amygdalin
B1 L-arabinose	B2 D-arabitol	B3 arbutin	B4 cellobiose	B5 D-fructose	B6 L-fucose	B7 D-galactose	B8 D-galacturonic acid	B9 gentiobiose	B10 D-gluconic acid	B11 α-D-glucose	B12 m-inositol
C1 α-D-lactose	C2 lactulose	C3 maltose	C4 maltotriose	C5 D-mannitol	C6 D-mannose	C7 D-melezitose	C8 D-melibiose	C9 α-methyl D-galactoside	C10 β-methyl D-galactoside	C11 3-methyl glucose	C12 α-methyl D-glucoside
D1 β-methyl D-glucoside	D2 α-methyl D-mannoside	D3 palatinose	D4 D-psicose	D5 D-raffinose	D6 L-rhamnose	D7 D-ribose	D8 salicin	D9 sedoheptulosan	D10 D-sorbitol	D11 stachyose	D12 sucrose
E1 D-tagatose	E2 D-trehalose	E3 turanose	E4 xylitol	E5 D-xylose	E6 acetic acid	E7 α-hydroxybutyric acid	E8 β-hydroxybutyric acid	E9 γ-hydroxybutyric acid	E10 p-hydroxyphenyl acetic acid	E11 α-keto glutaric acid	E12 α-keto valeric acid
F1 lactamide	F2 D-lactic acid methyl ester	F3 L-lactic acid	F4 D-malic acid	F5 L-malic acid	F6 methyl pyruvate	F7 mono-methyl succinate	F8 propionic acid	F9 pyruvic acid	F10 succinamic acid	F11 succinic acid	F12 N-acetyl L-glutamic acid
G1 alaninamide	G2 D-alanine	G3 L-alanine	G4 L-alanyl-glycine	G5 L-asparagine	G6 L-glutamic acid	G7 glycyl-L-glutamic acid	G8 L-pyroglutamic acid	G9 L-serine	G10 putrescine	G11 2,3-butanediol	G12 glycerol
H1 adenosine	H2 2'-deoxy adenosine	H3 inosine	H4 thymidine	H5 uridine	H6 adenosine-5'-monophosphate	H7 thymidine-5'-monophosphate	H8 uridine-5'-monophosphate	H9 fructose-6-phosphate	H10 glucose-1-phosphate	H11 glucose-6-phosphate	H12 D-L-α-glycerol phosphate

FIG. 2. BIOLOG plate indicating substrates used in this study.

A 1:100 slurry of soil in Ringer solution (Table 1) was prepared for each composite sample on the day of collection, and shaken vigorously for one minute to remove bacterial cells from the soil particles. Each BIOLOG cell well was

inoculated with 150 µl of the slurry, using a multi-channel pipetting device, and the plate was sealed in a sterile sampling bag. Plates were incubated at room temperature, and scored visually at 72 and 96 hours. The appearance of a purple color, due to the reduction of tetrazolium dye (oxygen depletion), indicated use of the carbon substrate in a particular well, and was considered a positive response.

TABLE 1. Chemical Constituents of Culture Media and Dilution Solutions

Bushnell-Haas Broth	
Potassium phosphate, monobasic	1.00 g/L
Potassium phosphate, dibasic	1.00 g/L
Ammonium nitrate	1.00 g/L
Magnesium sulfate, heptahydrous	0.20 g/L
Ferric chloride	0.05 g/L
Calcium chloride, dihydrate	0.02 g/L
Volume brought up to 1 L with distilled water.	
Ringer Solution	
Sodium chloride	2.150 g/L
Sodium thiosulfate pentahydrate	0.500 g/L
Calcium chloride, anhydrous	0.120 g/L
Potassium chloride	0.075 g/L
Volume brought up to 1 L with distilled water.	
Nutrient Broth	
Bacto Peptone	5.0 g/L
Bacto Beef Extract	3.0 g/L
Volume brought up to 1 L with distilled water.	

Heterotrophic Microorganism Enumeration

Microorganism numbers were measured from each soil sample using the modified, miniaturized, sheen screen Most Probable Number (MPN) method of Brown & Braddock (1990). Two sterile 24-well tissue culture plates were used for each sample. A 1:10 dilution of soil in Ringer solution was made, and serially diluted along the top row of eight wells by adding 0.2 mL of the slurry to 1.8 mL of sterile Difco Nutrient Broth (Difco Laboratories, Detroit, MI). The remaining wells contained approximately 2.0 mL of sterile Nutrient Broth. Each column of five wells was inoculated with a 100 μl aliquot of the dilution well contents using a multi-channel pipetting device. The plates were incubated for three days at room temperature, and wells were scored as positive if growth or turbidity was observed. Numbers of microorganisms were then calculated using a traditional five tube MPN index with 95% confidence limits, as reported in Standard Methods (Thomas, 1942).

Dry and Organic Weights

All soil samples were analyzed for dry weight and organic content. Water holding capacity and organic matter

content was determined gravimetrically (Black, 1965a; 1965b). Samples from each distance in the vegetative strips were dried at 105 °C for 24 hours.

For organic matter determination samples were ashed in a muffle furnace at 400 °C for 90 minutes, reweighed, and organic content was calculated.

Mycorrhizal Fungi

Mycorrhizal spores (*Gigaspora margarita*) grown on asparagus roots were obtained from the Dept. of Plant Pathology, KSU, Manhattan, Kansas. Spores were removed and sterilized using protocol described by Dr. Barbara Hetrick at the University of Northern Iowa (Personal Communication, 1995). Soil was rinsed with water, and seived through a 0.63 mm screen. The portion of soil retained on the seive was decanted off and subjected to sucrose density gradient centrifugation. Ten mL of 40%, 20%, and 10% were injected sequentially, using a syringe, into centrifuge tubes to establish density gradients. The portion of soil retained after sieving was slowly poured into the centrifuge tube, so as not to disturb the sucrose gradients, and centrifuged at 3,000 rpm's for 3 minutes. After centrifuging, spores were

removed from the gradient interfaces using a micropipette, and other debris was discarded. Individual spores were selected under a dissecting microscope using disposable pipettors, placed in 35 mL of Ringer solution and stored in the dark at 4 °C for approximately four months (Godfrey, 1957; Daniels and Graham, 1976).

The Ringer solution containing the spores was poured through a funnel lined with a #1 Whatmann filter paper (9.0 cm). Spores on the filter paper were surface sterilized using an aqueous solution containing 0.5% NaOCl (10% household bleach in distilled water). Sterile distilled water was used to rinse spores down to the bottom of the filter paper cone, and then the bleach solution was continually poured into the funnel to keep spores covered for three minutes. Spores were then removed from the filter paper, using sterilized forceps under a dissecting microscope, and placed onto prepared agar plates.

The ratio of Alachlor, Atrazine, and Acetachlor used for the first herbicide mixture application at the field site, was also used for evaluating the effect on mycorrhizal growth. Varying concentrations (0 ppm, 90 ppm, 180 ppm, 360

ppm, 620 ppm, 900 ppm, and 1800 ppm) of the mixture were added to 7.5 g/L agar, volume was brought up to 30 mL, and poured into deep agar plates (150 x 15 mm). Dialysis membrane was cut to the size of the agar plates, and autoclaved in a container of distilled water to keep them moist. The dialysis membrane was placed, using sterile forceps, on top of the agar in the petri plates, and ten mycorrhizal spores were placed on top of the dialysis membrane. Agar plates were sealed with parafilm and incubated at room temperature. Hyphal growth was measured after three and seven days incubation. A spore was considered to have germinated if at least one germ tube was observed extending from it.

Spore Formation Investigation

In an attempt to stimulate sporulation of mycorrhizal hyphae, solutions of 0.1 M adenosinetriphosphate (ATP) and 0.1 M cyclic adensinemonophosphate (cAMP) were added to spores placed on agar plates. Plates were incubated at room temperature through hyphae formation, until it was evident that spore formation was not going to occur (about 4 weeks).

Water Sampling

Samples for analysis of total suspended solids, organic nitrogen, nitrate, nitrite, Acetachlor, Alachlor, and Atrazine were obtained from 16 x 2 inch polyvinylchloride tubes located at each of the sampling distances. Pipes had a cap at one end, and were anchored in soil with the open end level with the soil surface to catch water flow. Wet chemistry analysis utilized standard methods described in Greenberg et al. (1992) for examination of water and wastewater. Nitrate, nitrite, and ammonium concentrations were analyzed using Dionex DX-100 Ion chromatograph, while detection of organic herbicide residues were analyzed using Waters High Performance Liquid Chromatograph (HPLC) equipment and Supelco (LC-8-supelcosil) columns. Results of these measurements are available in Krueger (Environmental Science Department, University of Northern Iowa).

RESULTS

Overview

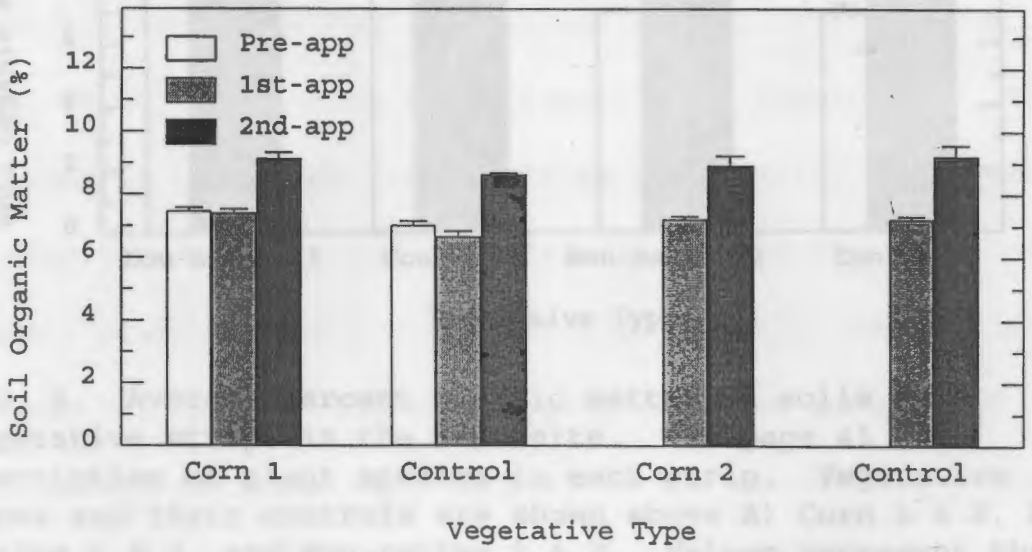
Data for this project, includes heterotrophic microorganism number, dry weights and organic matter content, mycorrhizal hyphal growth, and substrate utilization profiles in BIOLOG plates. Results presented were taken before and after two herbicide applications to an agricultural field. Applications correspond to the time of herbicide application to the field and are represented as pre-application (Pre-app.), first herbicide application (1st app.), and second herbicide application (2nd-app.). The Corn 2 strip was not developed until after initial sampling (Pre-app.), so all strips pertaining to Corn 2 will have one less data set. A complete tabulation of all data collected in this study is found in Appendices 1, 2, and 3.

Analysis of Organic Matter

Percentages of organic matter present in the first sample set ranged from 6.99-8.36% for various vegetative strips (Fig. 3). Averages in the 2nd sample set were higher, ranging from 7.20-9.28%. The third sample set contained the highest percentages of organic matter, ranging

from 9.00-9.41% across the vegetative types. In the Native 1 vegetative strip and its control, Non-native 1, Non-native 2 and its control strip, organic matter was lowest at the first sample time, and then increased over the next two sample times. Organic matter content of the Corn 2 strip increased from the second to third sample time. In the Corn 1 strip and its control, and Native 2 strip, organic matter was higher at the first sample time, than at the second, but highest at the third sample time. In nearly all the strips, organic matter was highest at the third sampling time.

a)



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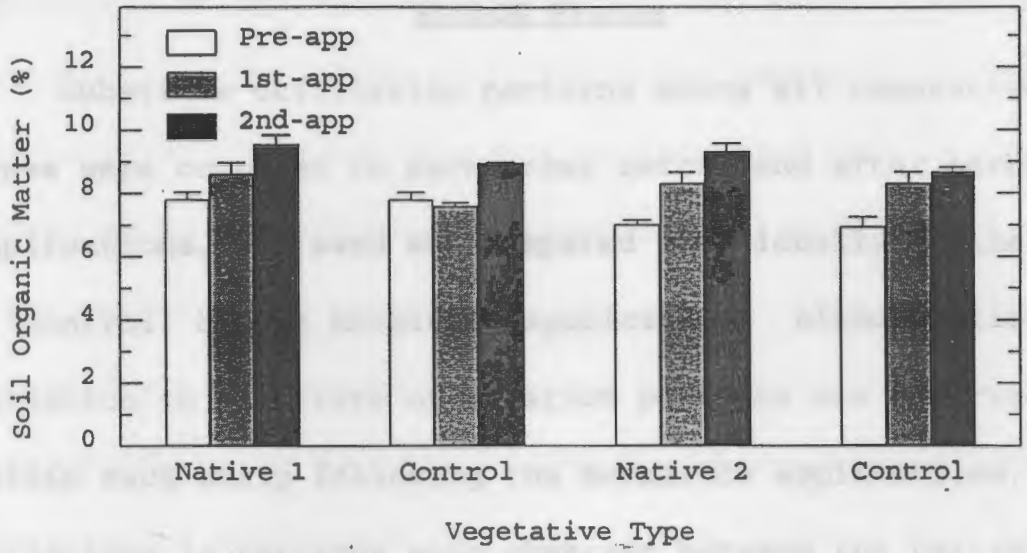

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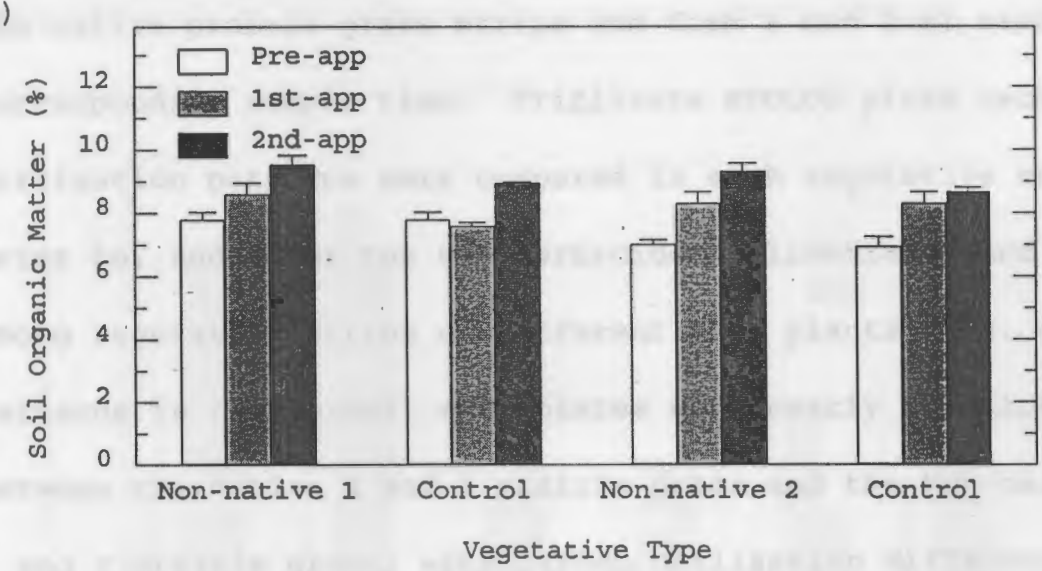


FIG. 3. Average percent organic matter in soils from vegetative strips in the test site. See page 41 for description of plant species in each strip. Vegetative types and their controls are shown above A) Corn 1 & 2, B) Native 1 & 2, and Non-native 1 & 2. Values represent the mean of 4 samples in the first quadrants (site of herbicide runoff) and 2 samples in the 4th quadrants (controls). Error bars represent ±1 standard deviation from the mean.

BIOLOG Plates

Substrate utilization patterns among all vegetative types were compared to each other before and after herbicide applications, and each was compared individually to the Corn 1 (control) before herbicide application. Although little variation in substrate utilization patterns was observed within each strip following the herbicide applications, variations in patterns were observed between the native and non-native prairie grass strips and Corn 1 nad 2 at each corresponding sample time. Triplicate BIOLOG plate carbon utilization patterns were compared in each vegetative strip, prior to, and after the two herbicide applications, and among vegetative strips of different host plants (Fig. 4). Patterns in the 95-cell well plates were nearly identical between the Native 1 and 2 prairie grass and the Non-native 1 and 2 prairie grass, with carbon utilization differences of only 2-3 cells. The native prairie grass strips had utilization patterns very similar to the non-native prairie grass strips at corresponding sampling times. Within the same prairie grass types, patterns changed very little from pre-herbicide application, through the two applications.

Again, variations were only seen in a few cells. Similarly, in Corn 1 and 2, carbon utilization patterns varied very little after the herbicide applications, and both strips had nearly identical patterns at corresponding sampling times. Large variation in patterns on the BIOLOG plates only observed when comparing native and non-native prairie grass strip patterns to both Corn 1 and 2 patterns. There were fewer carbon substrates utilized in Corn 1 and 2 than in both the native and non-native prairie grass strips (including the control quadrants).

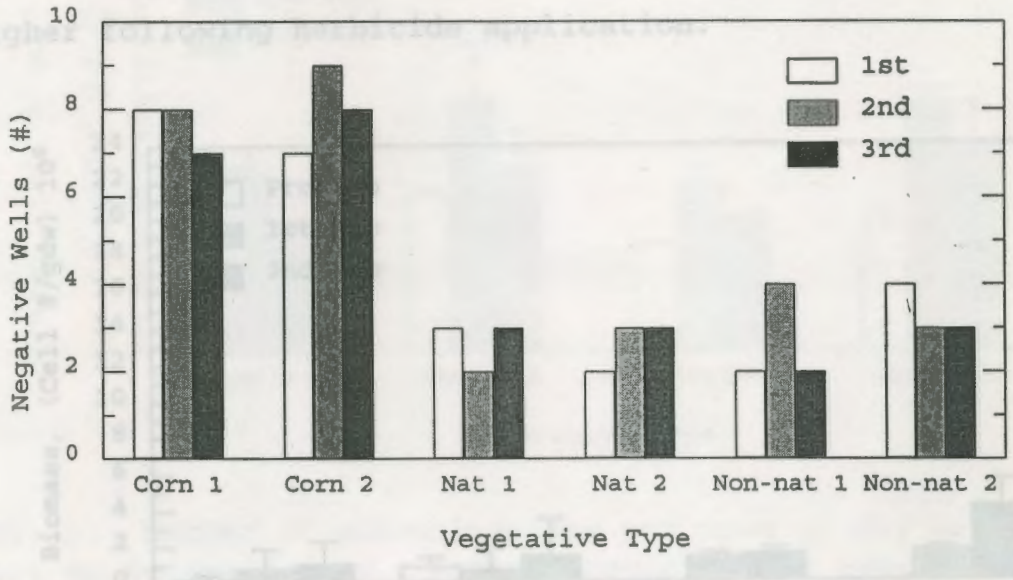


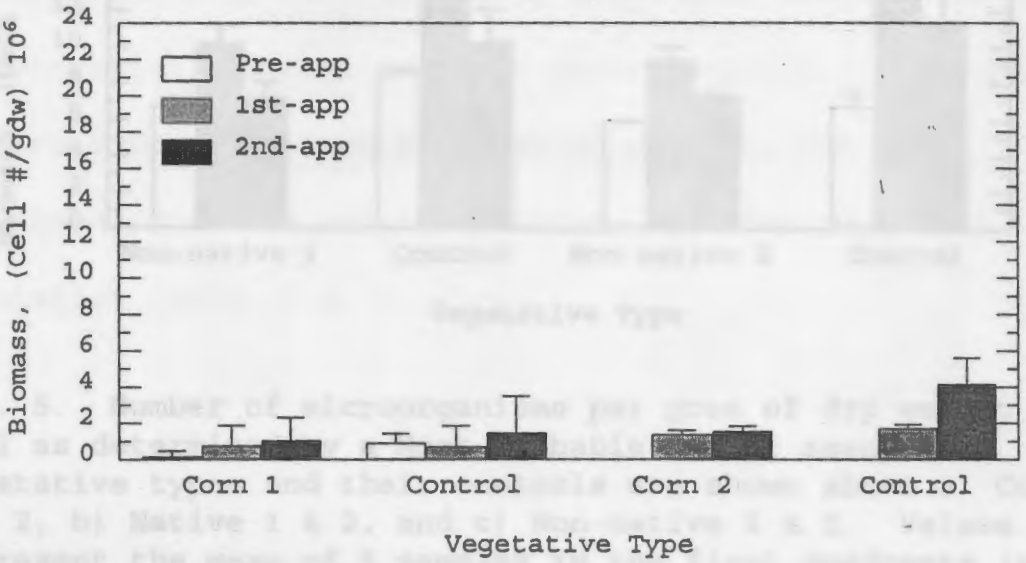
FIG. 4. Carbon substrate utilization patterns observed with BIOLOG plate assays compared among vegetative types, before and after herbicide applications. The Y-axis (negative wells) indicates the number of substrate not used by the microbial consortia after 3 and 4 days incubation. See Fig. 3 for description of vegetative types.

Microorganism Numbers

The Most Probable Numbers of microorganisms per gram of soil were averaged from composite samples made by blending three core samples from each sampling distance from the point of herbicide application (Fig. 5). High Performance Liquid Chromatograph analysis confirmed that herbicides in runoff water were not present in soil samples beyond the first 4.0 m (first quadrant; see Krueger, University of Northern Iowa, 1996).

In all the prairie strips, microorganism numbers were higher following herbicide application.

a)



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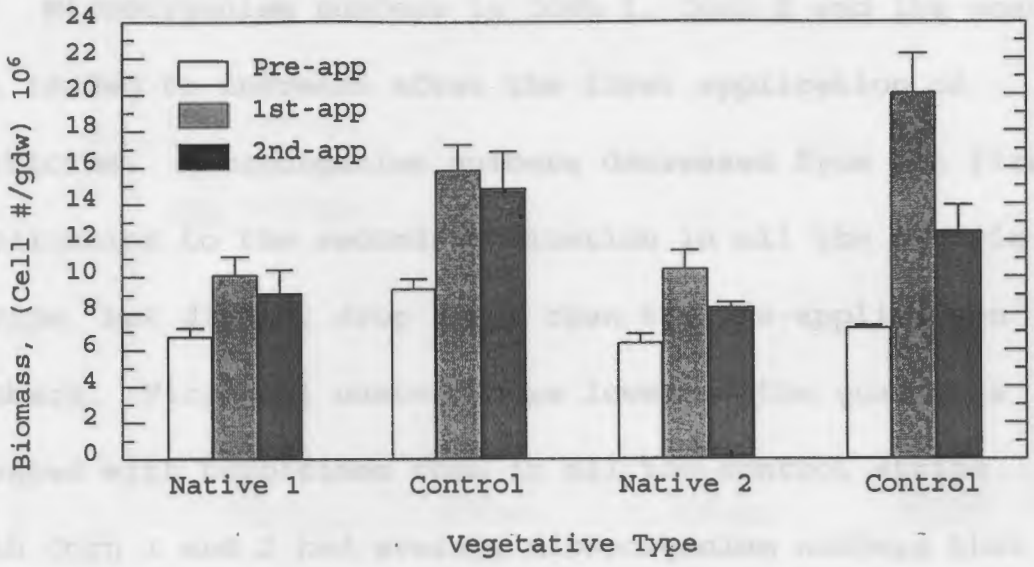

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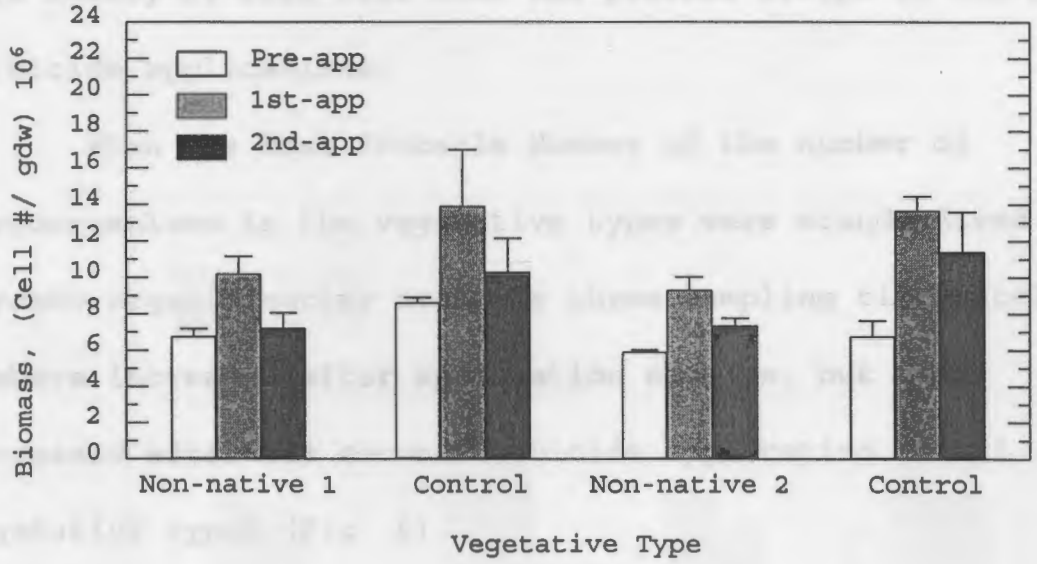
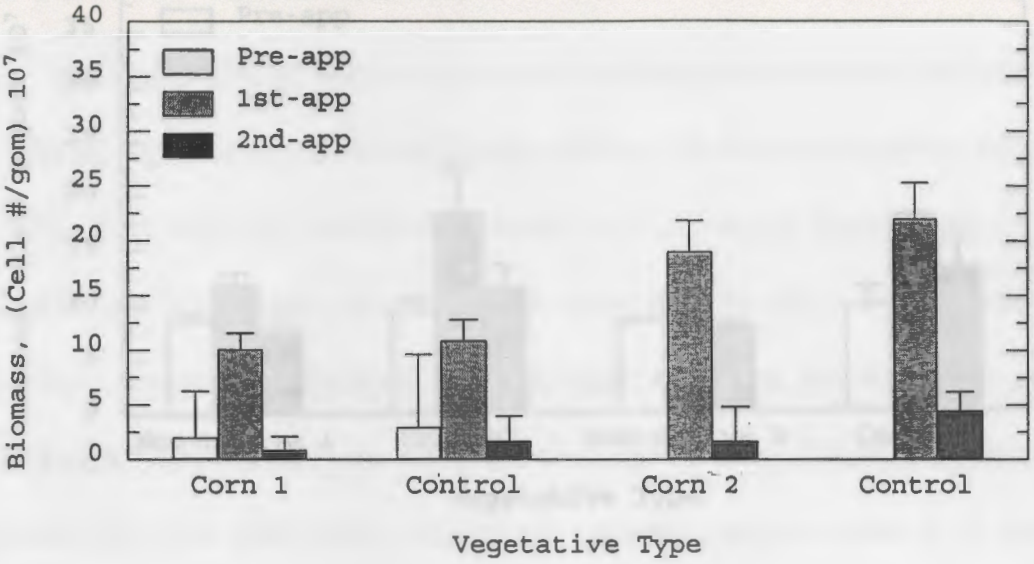


FIG. 5. Number of microorganisms per gram of dry weight soil as determined by a Most Probable Number assay. Vegetative types and their controls are shown above a) Corn 1 & 2, b) Native 1 & 2, and c) Non-native 1 & 2. Values represent the mean of 5 samples in the first quadrants (site of herbicide runoff) and 2 samples in the 4th quadrants (controls). Error bars represent ±1 standard deviation from the mean.

Microorganism numbers in Corn 1, Corn 2 and its control all tended to increase after the first application of herbicide. Microorganism numbers decreased from the first application to the second application in all the prairie strips, but did not drop lower than the pre-application numbers. Microbial numbers were lower in the quadrants treated with herbicides than in all the control strips. Both Corn 1 and 2 had average microorganism numbers that were nearly 10-fold less than the prairie strips at the same herbicide applications.

When the Most Probable Number of the number of microorganisms in the vegetative types were standardized to percent organic matter over the three sampling times, cell numbers increased after application samples, but then decreased after the second herbicide application in all vegetative types (Fig. 6).

a)



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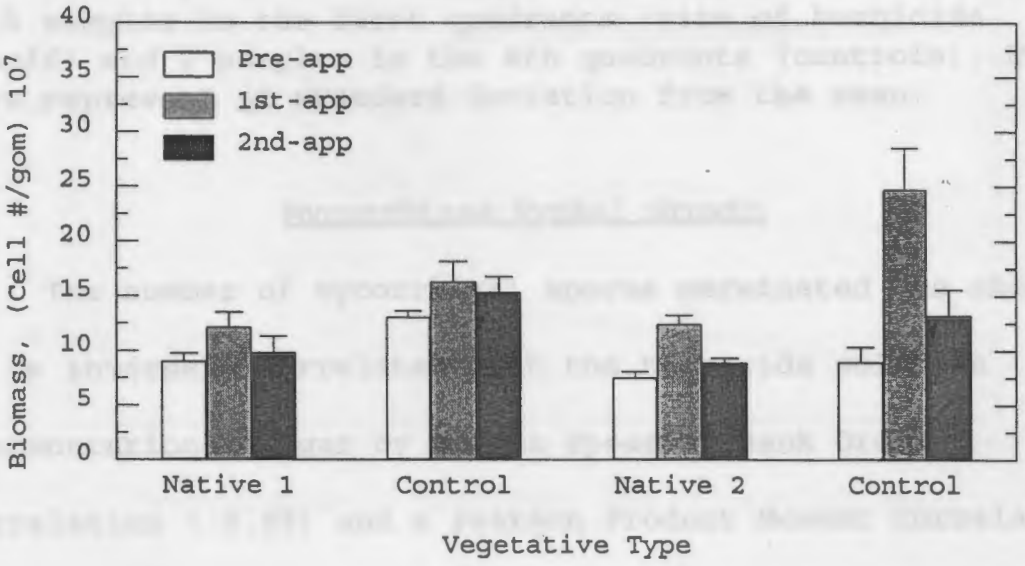

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c)

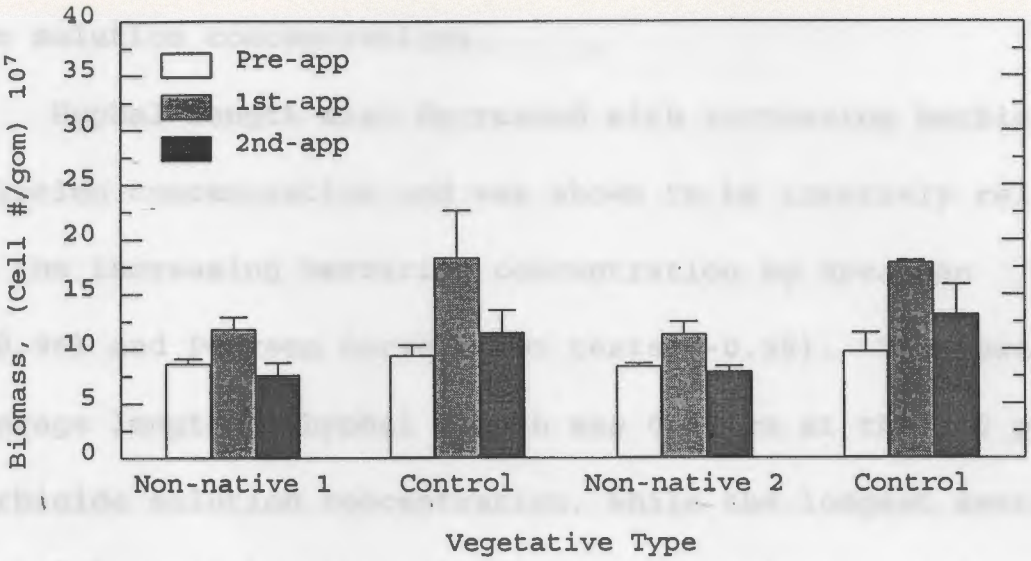

FIG. 6. Numbers of microorganisms in each vegetative type as a function of organic matter in soil. Vegetative types and their controls are shown above a) Corn 1 & 2, b) Native 1 & 2, and c) Non-native 1 & 2. Values represent the mean of 5 samples in the first quadrants (site of herbicide runoff) and 2 samples in the 4th quadrants (controls). Error bars represent ±1 standard deviation from the mean.

Mycorrhizae Hyphal Growth

The number of mycorrhizal spores germinated was shown to be inversely correlated with the herbicide solution concentration in agar by both a Spearman Rank Order Correlation (-0.98) and a Pearson Product Moment Correlation (-0.875; Fig. 7). The lowest number of germinated spores was observed at the 960 ppm herbicide solution

concentration, while the highest number was observed at 0.0 ppm solution concentrations.

Hyphal length also decreased with increasing herbicide solution concentration and was shown to be inversely related to the increasing herbicide concentration by Spearman (-0.96) and Pearson correlation tests (-0.95). The lowest average length of hyphal growth was 0.47 cm at the 960 ppm herbicide solution concentration, while the longest average hyphal length when the solution concentration was 0.0 ppm.

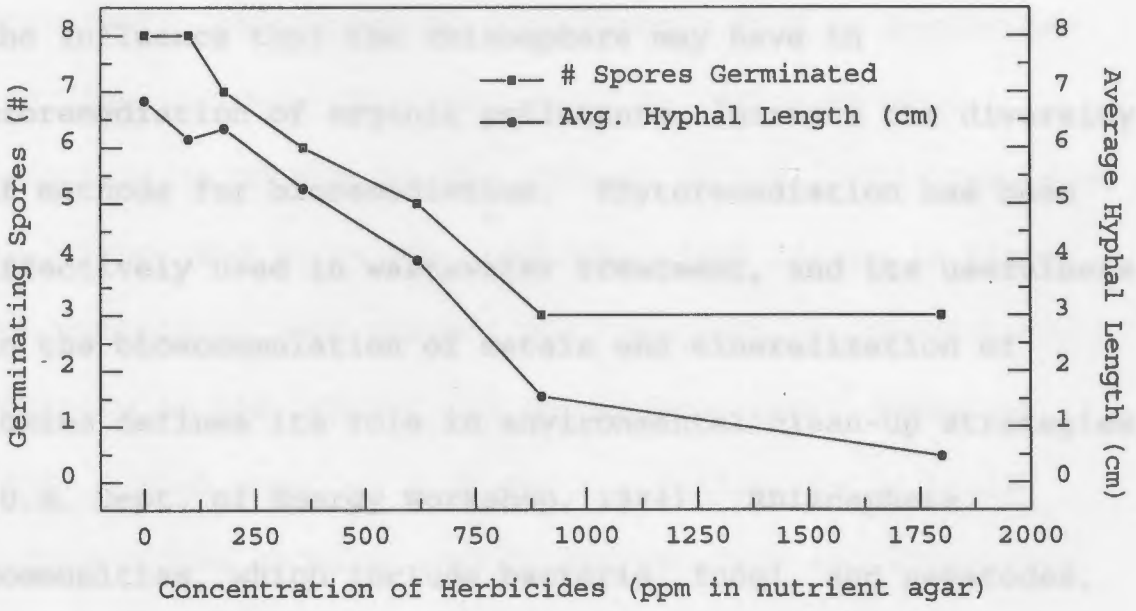

FIG. 7. Number of mycorrhizal spores which germinated and average mycorrhizae hyphal length as a function of herbicides concentration.

DISCUSSION

Overview

The use of living organisms to remediate potentially dangerous environmental pollutants is becoming an established method of treatment in an expanding array of situations. The natural capacity of organisms to degrade organic compounds offers a potential technology to deal with environmental hazards, by the developing new techniques and understandings of the processes involved (Atlas, 1995).

New areas of interest, such as phytoremediation, and the influence that the rhizosphere may have in bioremediation of organic pollutants, increase the diversity of methods for bioremediation. Phytoremediation has been effectively used in wastewater treatment, and its usefulness in the bioaccumulation of metals and mineralization of toxins defines its role in environmental clean-up strategies (U.S. Dept. of Energy Workshop, 1994). Rhizosphere communities, which include bacteria, fungi, and nematodes, have specifically been shown to increase the metabolic degradation of xenobiotic compounds (Anderson et al., 1993).

The changing activity of individual species within microbial consortia may influence entire ecological communities after an environmental stress. Thus changes in the activity of mycorrhizal fungi which increase root surface area of plants, and thus absorption of water and nutrients to their host may have profound effects on their plant host and associated rhizosphere microorganisms. Mycorrhizal fungi have been shown to degrade environmental contaminants such as atrazine (Kaufman and Blake, 1970), and polychlorinated biphenyls (Donnelly and Fletcher, 1994). Conversely, environmental contaminants such as herbicides and insecticides, have been shown to influence root colonization of mycorrhizae (Schwab et al., 1982; Trappe et al., 1994) and to be absorbed into plant tissue in higher concentrations than normal, via the fungi, increasing the toxicity of the contaminant to the host plant (Hamel et al., 1994). Such examples indicate the difficulty in truly understanding chemical and biological fate and effects of pollutants, and the need for methods that can evaluate the shifts and roles of such complex systems.

Although studies performed in the field may be more realistic than those done in a laboratory, variables are less controllable, so results are often subject to multiple interpretations. Perturbations to the environment can influence many areas of community structure, but inexpensive and accurate methods to evaluate effects are limited. Thus, overall impacts and potential recovery are hard to evaluate. It is this limitation that makes it necessary to improve methods to understand the effect of perturbations on community structure.

Molecular, biochemical, and metabolism based techniques to determine microbial structure and function are currently available. While some of these methods can give more insight into entire community responses than traditional methods, such as enumeration of microorganisms using plate counts (Frostegard et al., 1993), they still cannot determine how active individual strains and/or populations are in situ and how individual species interact (Bossio and Scow, 1995).

New techniques in population sampling, such as the BIOLOG plate method I chose to evaluate, must have the

potential to sample community structures easily and accurately. A rapid assessment method that could be used in field situations, and give reliable results of population shifts before and after environmental stress would be an effective monitoring tool. Recent publications have suggested that the BIOLOG plate method did have the potential to fill this need (Garland and Mills, 1991; Winding et al., 1993; Zak et al., 1994).

My hypothesis was that soil microbial composition would be characteristic of vegetative type in soil test strips and that changes would reflect herbicide perturbations to the field. Furthermore, the BIOLOG plate technique could be used as a rapid monitoring tool to characterize the population shifts through sole-carbon utilization patterns seen 72 and 96 hours after sampling was performed.

I additionally hypothesized that mycorrhizal hyphal growth would be negatively affected when grown on medium of increasing concentrations of herbicides, and that such a response could ultimately be used to characterize unknown perturbations to soil. This information could prove useful for assessing remediation strategies in the future.

Organic Matter

Results showed that percent organic matter tended to increase throughout the three sampling times. This may be explained by an influence of increased temperature and rainfall over the growing season. Soil is a very complex and heterogenous medium which may account for variations within the treatment types during sample times.

BIOLOG Plates

Carbon utilization pattern results indicated that microbial populations were very similar in all the prairie grass strips, and dramatic population shifts were not evident after the two herbicide applications. This suggests that herbicide application did not influence the structure of the microbial communities as measured by carbon utilization patterns. The BIOLOG plate results imply, however, that there is a difference in microbial community structure when comparing a monoculture (corn) with a mixture of native or non-native prairie plants. Corn 1 and 2 patterns had fewer substrates utilized than the prairie grass, suggesting that the corn had fewer microbial strains within its population to utilize the carbon sources. The

BIOLOG plate method only showed minor differences in utilization patterns, which indicates that the method would not be sensitive to small perturbations which limits its usefulness. Furthermore, with this method there is no way of telling if all microbial species are equally active in the utilization of the carbon substrates (e.g., which populations are most active in situ). Since the BIOLOG method is an enrichment technique, it is not possible to tell if all organisms are equally active in situ, or if shifts inactivity occur after a perturbation, unless an entire metabolic type disappears.

When plates were scored at 72 and 96 hours, definite patterns were seen, with changes noticed after the 72 hour incubation period. The BIOLOG plate method is based on the ability of microorganism to use a particular carbon substrate, but a positive or negative response can be seen depending on the time of scoring. This implies that the BIOLOG plate method is biomass dependent for its results, and that time of scoring is an important factor. After prolonged incubation, nearly every substrate was utilized, suggesting that positive reactions are not only dependent on

the microorganisms present, but how many of each type is present at the time of sampling.

While the BIOLOG plate method characterizes the ability of carbon substrate utilization in a population, it could be very much affected by the number of organisms capable of using a carbon source at a given time. For example, if only a few organisms capable of using arginine are present in a sample, but many more of the same organism are present in a second sample, the second sample will show a positive result on the microplate more quickly than the first. The first sample may not even show a positive result within the scoring period, suggesting a negative result, when in fact there were organisms capable of using that substrate, just very few initially.

My observation that this sampling method is very biomass dependent, limits its usefulness as a rapid assessment tool and should not be considered a complete monitoring assay for environmental samples. To further test if differences in carbon utilization was based on community structure or population numbers, biomass was also compared among the vegetative strips.

Microorganism Numbers

Microorganism numbers were compared among the various vegetative types, and between quadrants receiving herbicide runoff and their controls using a modified five tube MPN method. Numbers as a function of vegetative type were also evaluated between quadrants receiving herbicide runoff and their controls.

After observing the increase in percent organic matter over the three sampling times, one might expect that cell numbers would increase over the sampling times as well. This was not seen after the 2nd herbicide treatment in most strips, suggesting that the strong application had a negative effect on the number of microorganisms in the soil.

When cell numbers were standardized to percent organic matter, a decrease was seen in all the vegetative types after the 2nd herbicide treatment. Microorganism numbers increased from the first sampling time to the second in all the prairie grass strips, and then numbers decreased at the third sampling time. This data strongly reinforces the idea that the second herbicide application had a negative effect on the number of heterotrophic microorganisms in the soil,

if not their overall ability to degrade a variety of carbon substrates. The effect seen was a decrease in the number of organisms present in the soil, and subsequently, the rate that organic contaminants could potentially be degraded by those populations.

Mycorrhizae Hyphal Growth

Results of my study seem to support those of Trappe et al. (1984) by decreasing the formation of hyphae. Other studies including one by Tsai and Philips (1991) showed that the addition of flavenoids and other root exudates can stimulate the growth of mycorrhizal spores *in vitro*. Mycorrhizal fungi can accumulate herbicides, such as Atrazine and 2,4-D, into their biomass, and some species are capable of mineralizing them (Kaufman and Blake, 1970; Donnelly et al., 1993). Donnelly et al. (1993) showed that certain mycorrhizae were able to tolerate high concentrations of Atrazine and 2,4-D in cultures, and also accumulate it in their biomass. Nitrogen concentration was shown to affect the amount of herbicide taken up by the mycorrhizal fungi, with more herbicide being taken up as nitrogen concentration was increased. Results from this

study seem to support those results, since mycorrhizal spores were able to germinate and produce hyphae of several centimeters in varying concentrations of the herbicide mixture.

The addition of ATP and cAMP to germinating hyphae did not induce spore formation in my study, which supports the fact that nobody has been able to induce spore formation in a culture. The question of when and why mycorrhizae are sometimes stimulated or not affected by the addition of organic contaminants, and other times are negatively affected by the addition is an area that would benefit from further research.

Summary

The purpose of this research was to test the effect of herbicides on microbial populations in field samples, and the growth of mycorrhizal hyphae. The remediation of contaminated soils through plants and indigenous organisms is becoming routine in many situations, thus it is crucial to understand the effects of contaminants on these populations. Chemical contaminants which alter populations useful in bioremediation should be identified and replaced

with those that are not harmful to the organisms that can mineralize or accumulate them. Emerging techniques, such as using Polymerase Chain Reaction to identify mycorrhizal spores from soil samples through the development of DNA primers, will improve the detection of mycorrhizal species so negative effects can be readily detected (Abbas and Jurgenson, 1996).

The BIOLOG plate technique was also tested to evaluate its effectiveness as a rapid monitoring tool for environmental sampling. Although varying patterns were seen between corn and prairie grasses, the BIOLOG method did not seem sensitive enough to detect small changes in population shifts after herbicide application. This method would not be useful for assessing affects of perturbations by organic pollutants and a subsequent remediation protocol. Time and biomass were discovered to be important factors to consider when using this technique, and while standardizing cell density in soil samples would decrease the importance of these factors, the method would no longer be considered a easy and rapid environmental monitoring tool. The BIOLOG method is a useful technique in characterizing microbial

populations, but the need for an effective way to evaluate complex and diverse ecological systems still exist.

Herbicide application was shown to decrease microbial numbers in vegetative filter strips, especially when standardized to the increase in organic matter over the same sampling period. There was also a difference seen in the microbial populations between vegetative strips of prairie grass mixtures and a monoculture of corn. Differences were observed among the strips in cell numbers, which were much lower in the monoculture of corn, and carbon utilization patterns.

Increasing concentrations of herbicides showed an effect on mycorrhizal fungi, decreasing germination and hyphal length. Spore formation was not accomplished with the techniques used in this study.

Efforts to maximize agricultural output through the increasing use of farm chemicals, result in damage to plants and indigenous microorganisms in that environment. Common farming practices influence the ability of an area to rebound after a perturbation, decreasing the ability of those organisms to mineralize or accumulate organic

pollutants. To accomplish sustainable farming practices well into the future, it would be wise to incorporate methods which not only spare natural populations, but use them to mineralize chemicals that are applied and even increase agricultural output, such as mycorrhizal fungi which increase absorbtion of minerals, waters and nutrients. To fully understand impacts on the natural environment, improved methods of characterizing and evaluating biological systems need to be found. Only then can we better understand complex population interactions among microorganisms and plants, preventing disturbances so they may help us achieve a healthier environment.

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APPENDIX A RAW DATA FOR ORGANIC CONTENT AND CELL NUMBERS

CORN 1 STRIP

% Organic Matter

Distance (m)	0.0	0.5	1.0	2.0	4.0	8.0	16.0	Average
pre	7.65	7.72	7.52	7.17	7.26	7.15	6.91	7.34
1st	7.60	7.61	7.11	7.54	7.17	6.51	6.83	7.20
2nd	9.73	9.04	9.29	9.04	8.59	8.68	8.60	9.00

Cell #/gdw soil (x E+06)

Distance (m)	0.0	0.5	1.0	2.0	4.0	8.0	16.0	Average
pre	0.08	0.13	0.16	0.13	0.26	0.27	0.17	0.17
1st	0.57	0.88	0.92	0.40	0.96	0.84	0.61	0.74
2nd	1.08	0.84	1.27	0.53	0.84	1.29	1.69	1.08

CORN 2 STRIP

Distance (m)

1st	0.0	0.5	1.0	2.0	4.0	8.0	16.0	Average
2nd	7.01	7.39	7.03	7.35	7.33	7.42	7.89	7.34
	8.71	9.73	8.10	9.47	8.62	9.56	8.88	9.01

Cell #/gdw soil (x E+06)

Distance (m)

1st	0.0	0.5	1.0	2.0	4.0	8.0	16.0	Average
2nd	1.32	1.32	2.56	2.53	2.59	2.58	3.76	2.38
	1.18	0.78	1.61	2.16	2.14	2.73	5.60	2.31

NATIVE 1 STRIP

% Organic Matter

Distance (m)

pre	0.0	0.5	1.0	2.0	4.0	8.0	16.0	Average
1st	7.58	7.65	7.33	7.64	7.24	7.25	7.00	7.39
2nd	8.43	8.63	9.59	10.01	8.84	9.98	9.50	9.28
	9.44	8.48	9.65	9.90	9.06	9.18	10.06	9.40

Cell #/gdw soil (x E+06)

Distance (m)

pre	0.0	0.5	1.0	2.0	4.0	8.0	16.0	Average
1st	5.80	6.07	7.05	6.37	8.38	9.87	8.80	7.48
2nd	11.10	11.40	8.80	9.06	14.19	14.47	17.24	12.32
	10.80	8.40	8.43	5.30	12.66	12.85	16.87	10.76

Continued on next page.

NATIVE 2 STRIP

% Organic Matter

Distance (m)	0.0	0.5	1.0	2.0	4.0	8.0	16.0	Average
pre	8.19	8.83	8.44	8.45	8.51	7.98	8.11	8.36
1st	7.96	7.39	7.41	9.00	9.92	8.52	7.80	8.28
2nd	9.22	9.36	10.03	9.20	8.97	10.14	8.94	9.41

Cell #/gdw soil (x E+06)

Distance (m)	0.0	0.5	1.0	2.0	4.0	8.0	16.0	Average
pre	6.76	8.05	6.14	5.72	5.32	8.26	6.19	6.63
1st	9.36	8.73	8.79	13.91	11.44	17.94	22.24	13.20
2nd	7.99	8.65	7.64	9.04	8.35	11.13	13.92	9.53

NON-NATIVE 1 STRIP

% Organic Matter

Distance (m)	0.0	0.5	1.0	2.0	4.0	8.0	16.0	Average
pre	7.42	7.77	8.06	7.80	8.49	9.33	8.60	8.21
1st	8.05	9.42	9.17	7.59	8.82	7.70	7.51	8.32
2nd	9.91	9.87	8.75	9.19	9.94	8.95	9.03	9.38

Cell #/gdw soil (x E+06)

Distance (m)	0.0	0.5	1.0	2.0	4.0	8.0	16.0	Average
pre	6.26	6.30	8.36	6.30	6.67	8.36	8.90	7.31
1st	12.00	11.09	8.61	7.36	11.99	10.83	17.00	11.27
2nd	6.00	8.27	10.01	5.79	6.06	8.49	12.15	8.11

NON-NATIVE 2 STRIP

% Organic Matter

Distance (m)	0.0	0.5	1.0	2.0	4.0	8.0	16.0	Average
pre	7.14	6.53	7.35	6.92	7.14	7.24	6.64	6.99
1st	7.92	7.53	9.37	8.93	7.78	7.12	8.16	8.12
2nd	8.50	9.76	9.58	9.78	8.96	8.80	8.55	9.13

Cell #/gdw soil (x E+06)

Distance (m)	0.0	0.5	1.0	2.0	4.0	8.0	16.0	Average
pre	5.75	5.95	5.69	5.85	6.35	5.90	7.63	6.16
1st	11.53	8.80	7.18	9.70	9.46	12.90	14.45	10.57
2nd	8.05	6.86	8.05	7.82	6.12	9.26	13.59	8.54

APPENDIX B RAW DATA FOR MYCORRHIZAL HYPHAL GROWTH AND GERMINATION

Herbicide Concentration (ppm)	# Spores Germinated	Avg. Hyphal Length (cm)
1800	3	0.47
900	3	1.54
620	5	3.99
360	6	5.27
180	7	6.34
100	8	6.15
0	8	6.83